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14. ABSTRACT

Myeloproliferative neoplasms (MPNs) are blood cancers that affect almost 300,000 people in the United States. MPN drugs (JAK inhibitors) do not effectively induce remission. Thus there is continued need to develop effective therapies for these blood cancers. The purpose of this work is to determine the effect of statins alone and in combination with JAK inhibition on MPN cells driven by the *JAK2-V617F* oncogene. We have determined that statins have growth inhibitory effects on MPN cells and induce cell death by apoptosis, which is prevented by the addition of mevalonate. Statin treatment also inhibited the colony formation of primary cells from MPN patients, but not from healthy individuals. Statin treatment alone or in combination with a JAK2 inhibitor did not affect disease formation in an MPN mouse model, suggesting statin effects on MPN cells may be restricted to *in vitro* experiments, or that the effects of statins on MPN cells *in vivo* requires doses of drug that have not been tested or are unattainable. While evidence that cholesterol plays a role in MPNs continues to mount, our work suggests additional research is required to determine if statins should be considered in a potential therapeutic strategy for MPNs.

15. SUBJECT TERMS
myeloproliferative neoplasm, MPN, JAK2, JAK2-V617F, statin, therapy, therapeutic

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1. INTRODUCTION:

Myeloproliferative neoplasms (MPNs) are blood cancers that affect almost 300,000 people in the United States (1). A mutant protein, JAK2-V617F, is an important factor in the development of these diseases as it is present in the majority of patients with MPNs (2). Targeting this mutant protein has been the goal of much research and recently a JAK inhibitor, ruxolitinib, was approved for use in MPNs. Unfortunately JAK inhibitors like ruxolitinib are unable to significantly reduce the number of diseased cells in patients and thus are unable to offer hope for remission (3). Thus, there is continued need to develop effective therapies for these blood cancers. One approach that has gotten much attention is to develop combination therapies of a JAK inhibitor and another agent. We recently, demonstrated that the cell signaling activity of JAK2-V617F, which causes most MPNs, is dependent on lipid rafts in the cell membrane (4). Lipid rafts are small regions of the membrane that are rich in cholesterol and other lipids (5). Depleting cholesterol from the rafts disrupts JAK2-V617F signaling (4). Utilizing a statin to decrease cellular cholesterol, we demonstrated that the growth of MPN cells driven by JAK2-V617F is sensitive to cholesterol-lowering drugs (4). As statins are widely used to control hypercholesterolemia (6), it is possible that combination of a JAK inhibitor with a statin may provide a more efficacious therapy than JAK inhibitor alone. The purpose of the research performed was to determine the extent to which statins can cooperate with JAK2 inhibition in combination therapy of MPN cells and to determine the extent to which combination therapy of statins and JAK2 inhibition can inhibit MPN formation in a mouse model.

2. KEYWORDS:

myeloproliferative neoplasm, MPN, JAK2, JAK2-V617F, statin, therapy, therapeutic

3. ACCOMPLISHMENTS:

What were the major goals of the project?

1. To determine the extent to which statins can cooperate with JAK2 inhibition in combination therapy of MPN cells.
2. To determine the extent to which combination therapy of statins and JAK2 inhibition can inhibit MPN formation in a mouse model.

What was accomplished under these goals?

In Goal 1, our studies were designed to determine the extent to which statins can cooperate with JAK2 inhibition in combination. This includes determining the efficacy of statins toward MPN model cell killing and growth and to determine statin effects on growth inhibition and cell killing of primary MPN cells. Our first experiments were designed to determine if there was a statin that was most efficacious against JAK2-V617-driven MPN model cells. We found that simvastatin (Fig. 1), and lovastatin (Fig. 2) each could inhibit the growth of MPN model cells, including HEL and Set2 cells, which are dependent on JAK2-V617F for growth. From this data we have identified simvastatin as the most efficacious statin toward inhibiting the growth of MPN model cells.

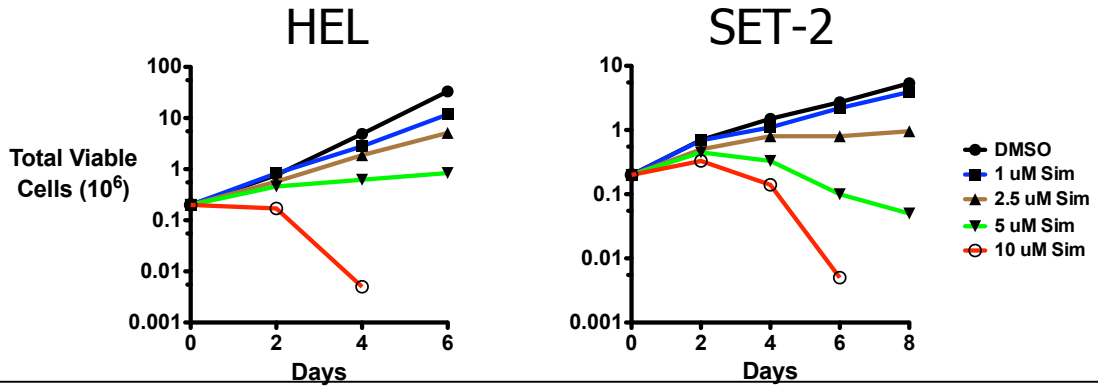

Figure 1: Effect of simvastatin on MPN model cell line growth. HEL and Set2 cells were incubated with varying concentrations of simvastatin. Total viable cells were determined by trypan blue over time. Simvastatin exhibited a dose dependent growth inhibitory effect on both cell lines.

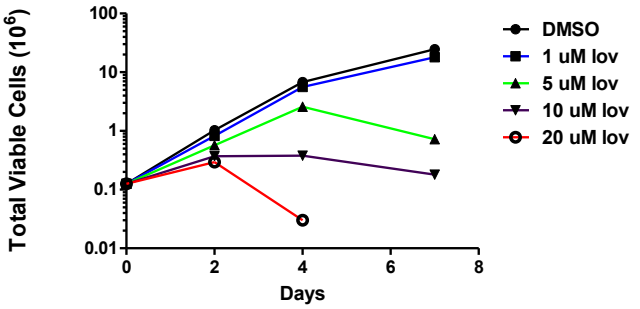

Figure 2: Effect of lovastatin on MPN model HEL cell line growth. HEL cells were incubated with varying concentrations of lovastatin. Total viable cells were determined by trypan blue over time. Lovastatin exhibited a dose dependent growth inhibitory effect on both cell lines.

Simvastatin reduced cell viability of HEL cells, which are driven by JAK2-V617F, but not K562 cells, which are driven by the BCR-ABL tyrosine kinase (Fig. 3). This suggests there may be specificity toward which oncogene-driven growth is affected by simvastatin.

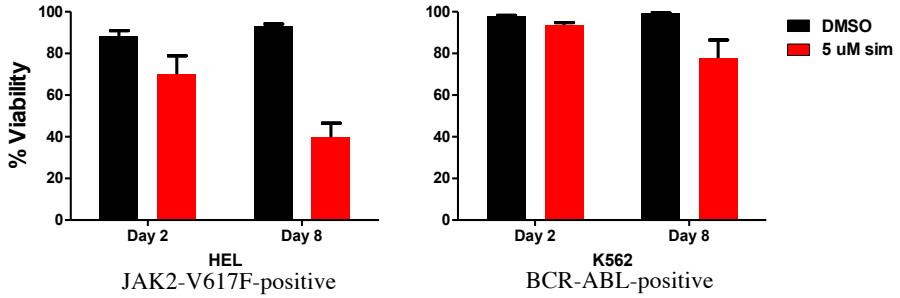

Figure 3: Effect of Simvastatin on HEL and K562 cell viability. HEL and K562 cells were incubated with 5 uM simvastatin. Cell viability was determined by trypan blue after two and eight days. Simvastatin decreased the viability (prominent at day 8) of the JAK2-V617F-driven HEL MPN model cell line but not the BCR-ABL-driven K562 myeloid cell line.

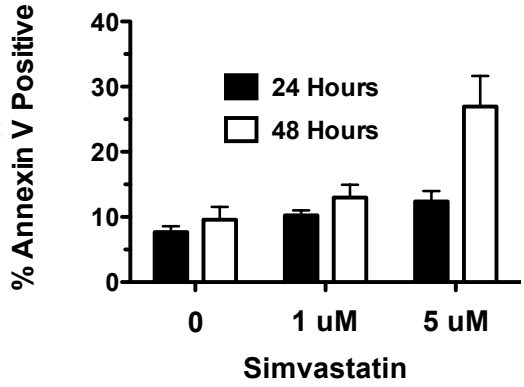

Figure 4: Effect of Simvastatin on HEL Cell Apoptosis. HEL cells were incubated with 1 uM and 5 uM simvastatin. Apoptosis was measured by Annexin V staining followed by flow cytometry after 24 and 48 hours. Apoptosis was detected at the higher simvastatin dose after 48 hours of treatment.

Statins inhibit the activity of HMG-CoA Reductase, which results in inhibition of mevalonate synthesis (6). Therefore, in order to determine if the statin affect observed on JAK2-V617F-dependent cells was due to the expected mechanism of HMG-CoA Reductase inhibition we performed mevalonate add-back experiments. Mevalonate add-back completely prevented the simvastatin effects on HEL cell growth (Fig. 5). This demonstrates that the statin effect observed is indeed due to the expected mechanism of action, that is, inhibition of HMG-CoA Reductase.

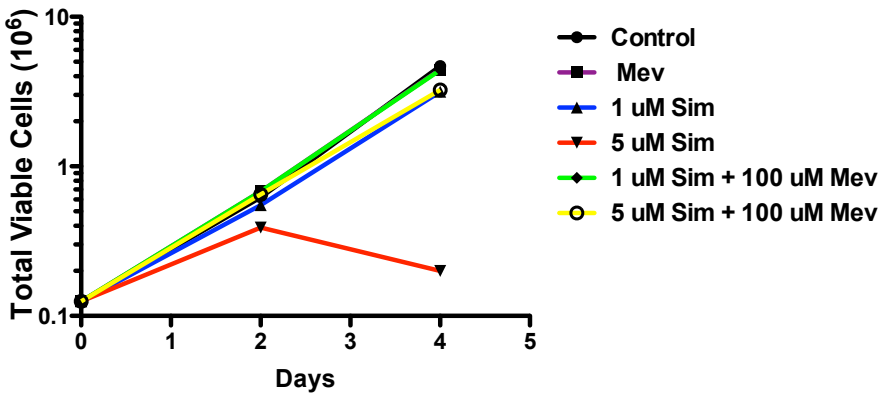

Figure 5: Mevalonate rescues HEL cells from the growth inhibitory effect of simvastatin. HEL cells were incubated with 1 uM and 5 uM simvastatin in the presence or absence of 100 uM mevalonate. Total numbers of viable cells were determined by trypan blue exclusion over time. Mevalonate prevented the growth inhibition induced by 5 uM simvastatin suggesting that the mechanism of action of simvastatin is indeed as expected, that is. the inhibition of HMG-CoA reductase.

Myeloid progenitor cells from MPN patients form erythropoietin-independent erythroid colonies in methylcellulose (7). This is a hallmark of these cells and is used as an assay to test new therapies against MPN cells (8). Simvastatin was chosen as the most effective statin to pursue in these studies. Treating primary MPN patients' cells in this assay reduced the ability of myeloid progenitor cells to form erythropoietin-independent erythroid colonies in methylcellulose (Fig. 6A). Simvastatin did not affect colony formation from healthy controls (Fig. 6B). This result is important as it suggests statins may be able to affect aberrant signaling that leads to myeloid cell growth and differentiation in patients.

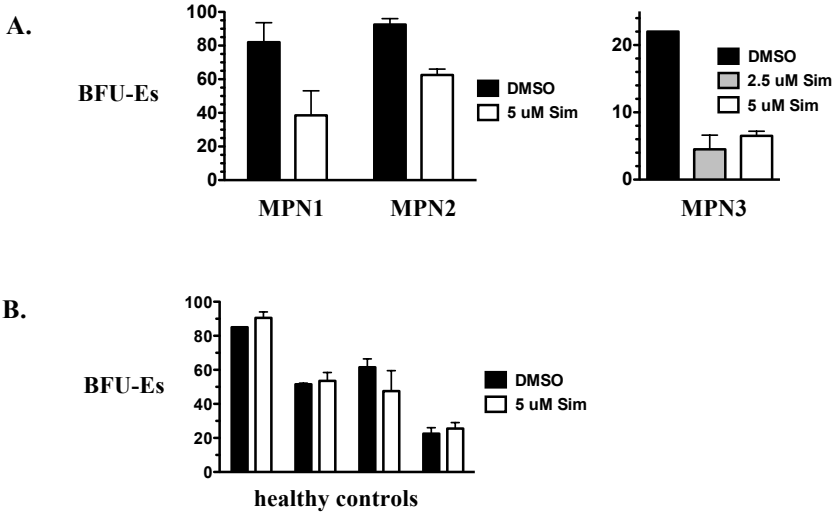

Figure 6: Statin treatment inhibits MPN patient progenitor cell colony formation. MPN progenitor cells from MPN patients form erythropoietin independent erythroid colonies in methylcellulose. A. Simvastatin inhibited this colony formation in three JAK2-V617F-positive MPN patients. MPN1 is a polycythemia vera patient and MPN2 and MPN3 are myelofibrosis patients. B. Simvastatin did not inhibit erythropoietin-dependent colony formation in healthy controls. This data suggests that statins may affect the growth properties of neoplastic myeloid progenitor cells in MPN patients and thus may be able to inhibit myeloproliferation in MPN patients. Error bars indicate standard deviation.

We next tested the ability of statin treatment to enhance the ability of JAK2 inhibitors to inhibit the growth of MPN model cell lines. Simvastatin together with the pan JAK inhibitor, JAK inhibitor I, had an impressive synergistic effect against HEL cells, effectively preventing these cells from growing in culture (Fig. 7). This contrasts with the ability of these cells to continue to proliferate, albeit at reduced rates, when only a single drug was utilized. Similarly, simvastatin synergized with ruxolitinib (INCB018424) in HEL cells (Fig. 8), atorvastatin synergized with ruxolitinib (INCB018424) in HEL cells (Fig. 9), and lovastatin synergized with ruxolitinib (INCB018424) in HEL cells (not shown).

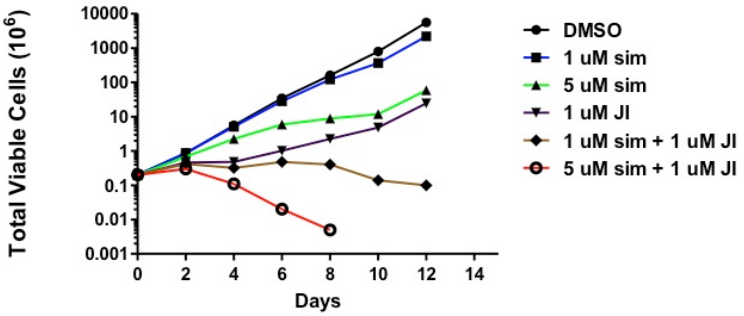

Figure 7: Simvastatin synergizes with JAK Inhibitor I to inhibit HEL cell growth. The MPN model, and JAK2-V617F-dependent, patient-derived cell line HEL was treated with simvastatin and the pan JAK inhibitor JAK inhibitor I, alone and in combination, as indicated. Total viable cells were determined by trypan blue exclusion over time. Addition of simvastatin to JAK inhibitor I treatment prevented the long-term growth survival of these cells. This suggests statin treatment may synergize with JAK2 inhibition in MPN cells.

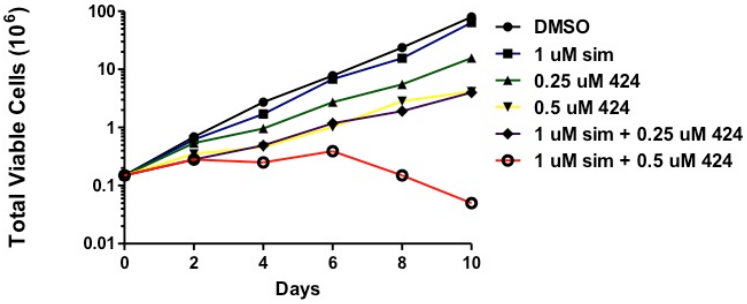

Figure 8: Simvastatin synergizes with ruxolitinib (INCB018424) to inhibit HEL cell growth. The MPN model, and JAK2-V617F-dependent, patient-derived cell line HEL was treated with simvastatin and ruxolitinib (INCB018424) (424), alone and in combination, as indicated. Total viable cells were determined by trypan blue exclusion over time. Addition of simvastatin to ruxolitinib (INCB018424) treatment prevented the long-term growth and survival of these cells. This suggests statin treatment may synergize with JAK2 inhibition in MPN cells.

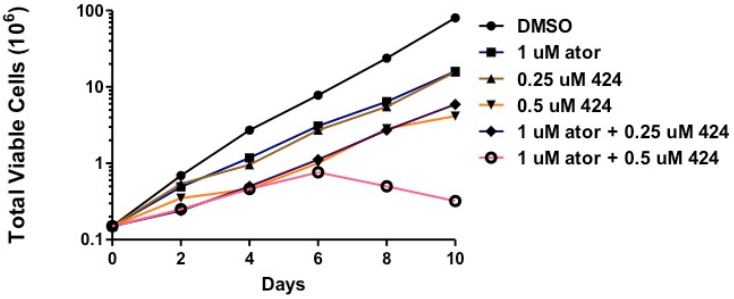

Figure 9: Atorvastatin synergizes with ruxolitinib (INCB018424) to inhibit HEL cell growth. The MPN model, and JAK2-V617F-dependent, patient-derived cell line HEL was treated with simvastatin and ruxolitinib (INCB018424) (424), alone and in combination, as indicated. Total viable cells were determined by trypan blue exclusion over time. Addition of simvastatin to ruxolitinib (INCB018424) treatment prevented the long-term growth and survival of these cells. This suggests statin treatment may synergize with JAK2 inhibition in MPN cells.

We tested the ability of statin treatment to enhance the ability of JAK2 inhibitors to induce apoptosis of MPN model cell lines. Simvastatin treatment enhanced the amount of HEL cell apoptosis over that induced by ruxolitinib (INCB018424) (Fig. 10). This suggests that combination of a statin with a JAK2 inhibitor may augment cell death induced by JAK2 inhibition alone.

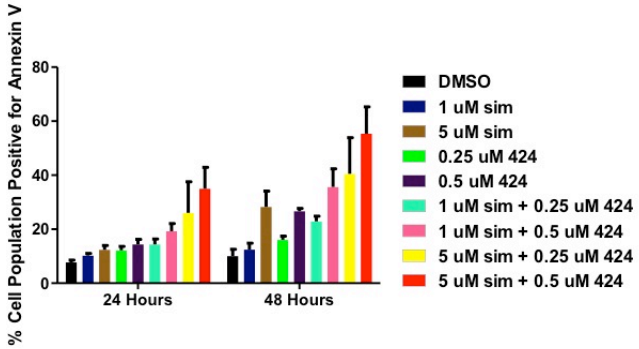

Figure 10: Simvastatin augments apoptotic cell death over that seen with JAK2 inhibitor treatment alone. The MPN model, and JAK2-V617F-dependent, patient-derived HEL cell line was treated with simvastatin and ruxolitinib (INCB018424) (424), alone and in combination, as indicated. Apoptosis was measured by annexin V staining and flow cytometry after 24 and 48 hours of treatment. Addition of simvastatin to ruxolitinib (INCB018424) treatment augmented apoptotic cell death over that seen with JAK2 inhibitor alone. This suggests combination statin treatment and JAK2 inhibitor treatment may result in more MPN cell death than JAK2 inhibitor treatment alone. Error bars indicate standard deviation.

We then tested the ability of combining statin and JAK2 inhibition in colony forming assays testing for erythropoietin-independent erythroid colony formation from MPN patients (as in Fig. 6). To do this we had to carefully select doses of simvastatin and the JAK2 inhibitor ruxolitinib that displayed sub-optimal/minimal effect as single agents. The inherent variability in utilizing primary cells from patients makes this complicated, because a single dose could have a wide varying effect on different samples. Nonetheless, we utilized 50 nM of ruxolitinib and 2.5 or 5.0 uM simvastatin for these studies. The data shown in Figure 11 suggests that while combining simvastatin and ruxolitinib displayed enhanced efficacy for inhibiting colony growth, this enhancement was marginal. The enhancement certainly did not display the level of synergy that we observed in MPN cell lines.

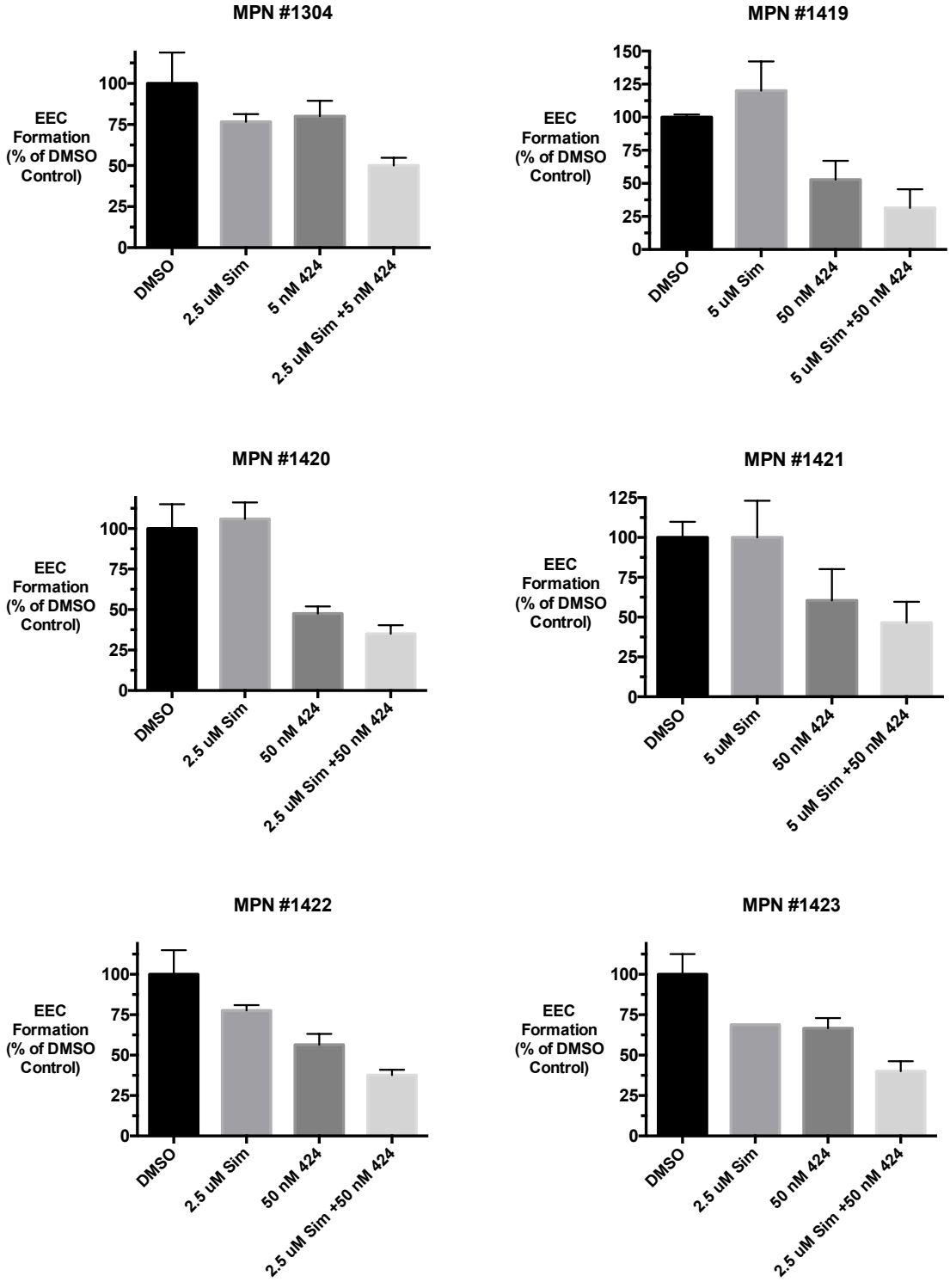

Figure 11: Simvastatin treatment does not significantly enhance inhibition of MPN patient erythroid colony formation by the JAK2 inhibitor ruxolitinib. MPN progenitor cells from MPN patients form erythropoietin-independent erythroid colonies in methylcellulose. Progenitor cells from MPN patients' peripheral blood were plated in methylcellulose in the absence of erythropoietin and erthyroid colonies were counted twelve days later. Cells were plated in the presence of 0.1% DMSO, 2.5 uM or 5 uM simvastatin (Sim), 50 nM ruxolitinib (424), and simvastatin and ruxolitinib together. These data suggest that statins in combination with ruxolitinib may not offer a therapeutic advantage over ruxolitinib therapy alone. Data are plotted as colony growth as a percent of the DMSO treated control sample. Error bars indicate standard deviation. Patients were JAK2-V617F-positive polycythemia vera (MPN #s 1304, 1419, and 1420), essential thrombocythemia (MPN #s 1421 and 1422), and myelofibrosis (MPN #1423).

In Goal 2, we have performed preliminary studies assessing the effects of statin mono-therapy in a MPN murine model. We initiated therapy 14 days after engraftment at which time mice had severe disease. Statin mono-therapy for 14 days did not result in a significant reduction in the white blood cell count, platelet count, or spleen size compared to animals treated with vehicle control. We also assessed the efficacy of longer-term treatment studies and did not detect a reduction in white blood cell count, platelet count, or spleen size compared to animals treated with vehicle control. This was a surprising result considering the impressive effect of statin treatment on MPN cells *in vitro*. We also assessed the ability of statin treatment to augment the effect of ruxolitinib treatment in an MPN animal model. Again, we saw no effect of statin treatment as described above.

With the same goal, we also tested a second MPN mouse model to determine if statin treatment could affect the ability of JAK2-V617F-driven cells to grow *in vivo*. For this experiment we utilized Balb/c mice injected with BaF3 cells that expressed JAK2-V617F and whose growth depended on JAK2-V617F activity. This is effectively the same mouse model system that was utilized to perform *in vivo* experiments with ruxolitinib before it was approved for use in MPN patients. In our experiments, we expressed the gene for firefly luciferase in the JAK2-V617F-dependent BaF3 cells so we could monitor cell growth and disease progression in live animal imaging experiments. Mice were imaged four days after injection of cells to confirm engraftment. Mice were treated with PBS or with 20 mg/kg simvastatin by oral gavage. Statin treatment did not affect growth of cells in mice as determined by *in vivo* imaging (Fig. 12).

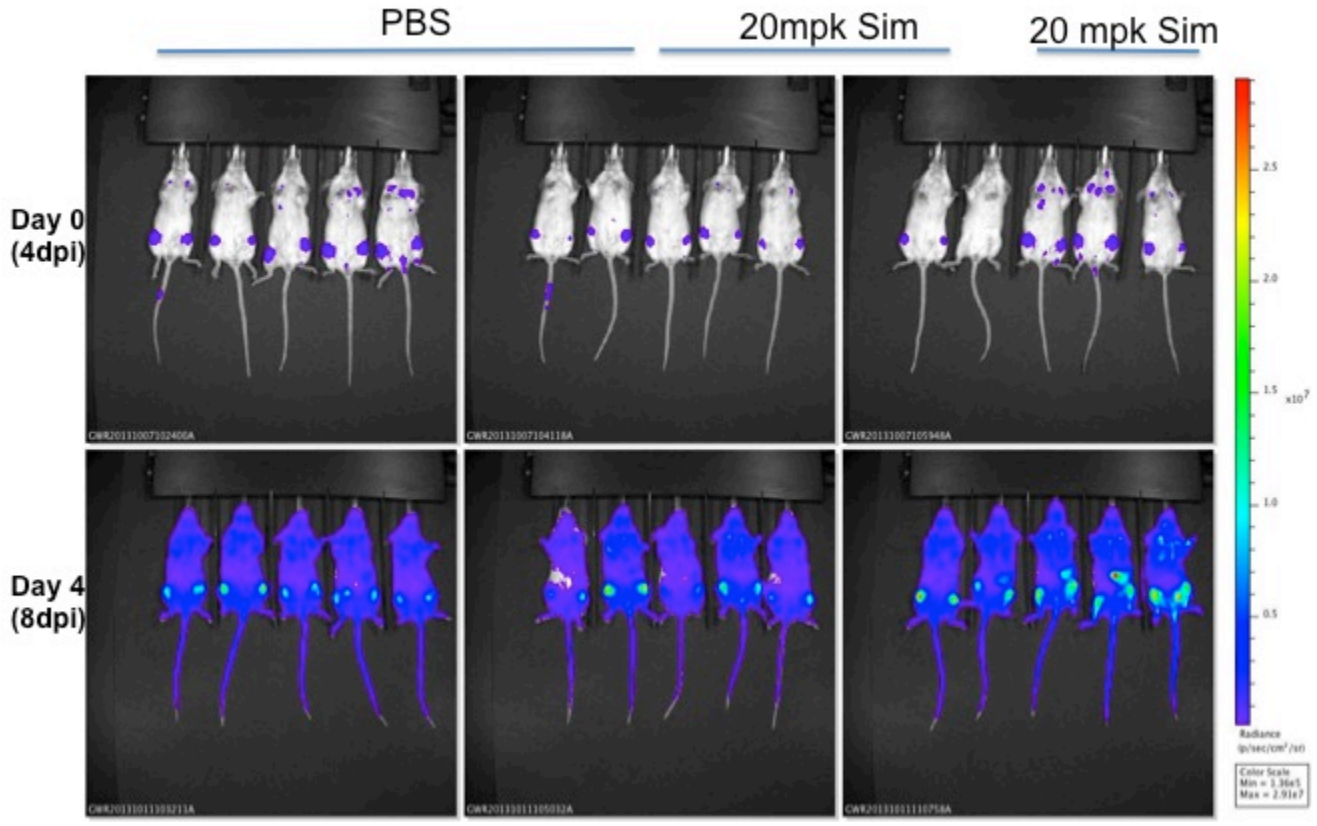

Figure 12: Simvastatin treatment does not affect JAK2-V617F-driven cell growth in mice. Mice injected with BaF3 cells expressing JAK2-V617F (and the gene for luciferase) and that are dependent on JAK2-V617F for growth were treated with PBS or 20 mg/kg simvastatin at four days post injection (dpi). Mice were imaged at four dpi and 8 dpi (following four days of treatment). Imaging indicates that mice treated with simvastatin had the same amount of JAK2-V617F cellular burden as mice treated with PBS, suggesting statin treatment had no effect on JAK2-V617F-dependent cell growth *in vivo*.

Summary of Key Research Accomplishments

-MPN model cells are sensitive to statin treatment.

-Simvastatin is more efficacious than other statins tested on growth inhibition of MPN model cells.

-Statin treatment decreases viability of MPN model cells.

-Statins induce apoptosis of MPN cells and this may be a mechanism by which they induce growth inhibition.

-The growth inhibition of MPN cells induced by statin treatment is prevented by mevalonate addition, suggestion the mechanism of action of statins against MPN cells is inhibition of HMG-CoA Reductase.

-Erythropoietin-independent growth of myeloid progenitors from MPN patients is inhibited by simvastatin.

-Statin treatment synergizes with JAK2 inhibitor treatment to inhibit growth of MPN model cells.

-Statin treatment in conjunction with JAK2 inhibitor treatment augments apoptotic cell death of MPN cells over JAK2 inhibitor treatment alone, suggesting such a combination may be a more efficacious therapy than JAK2 inhibitor treatment alone.

-Statin treatment did not significantly augment the ability of JAK2 inhibitor treatment to block erythropoietin-independent colony formation of primary cells of MPN patients.

-Statin treatment did not inhibit MPN formation in a mouse model, suggesting statins can not affect MPN formation or the dose of statin utilized did not reach a high enough level in the animals to affect MPN formation.

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What opportunities for training and professional development has the project provided?

-Lori Griner, a Ph.D. graduate student was awarded her Ph.D. degree during the first year of this award. Part of her training en route to her Ph.D. degree was during the time of funding of this grant. Lori Griner received a post-doctoral fellowship opportunity from the National Institutes of Health based on her training that was, in part, supported by this award. She is continuing her post-doctoral training in the same.

How were the results disseminated to communities of interest?

-Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

-Nothing to Report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

-The data obtained by performing the experiments funded by this grant suggests that statin treatment may improve JAK2 inhibitor therapy of MPN patients. Such an improvement is direly needed to take a step toward curing this disease. However, our studies in mice and with patient samples suggest perhaps other cholesterol lowering approaches in conjunction with JAK2 inhibitors should be considered over the use of statins. As other researchers have suggested that cholesterol may play a role in the development of MPNs, our work adds potentially important observations that dovetail nicely with this work of others.

What was the impact on other disciplines?

-Nothing to Report

What was the impact on technology transfer?

-Nothing to Report

What was the impact on society beyond science and technology?

-Nothing to Report

5. CHANGES/PROBLEMS:

-Nothing to Report

6. PRODUCTS:

-Nothing to Report (Although a future publication is likely, but has yet to be initiated.)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	*Gary Reuther*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Reuther was the principal investigator and scientific director of the project. He also performed colony forming assays and data analyses.*
Funding Support:	Also supported by institutional funds.

Name:	*Lori Griner*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Ms. Griner performed experiments involving statin and JAK2 inhibitor treatment of cells.*
Funding Support:	

Name:	*Que Lambert*
Project Role:	*Research Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Ms. Lambert assisted with the colony forming assays and the animal studies.*
Funding Support:	

Name:	*Anuradha Pradhan*
Project Role:	*Post-doctoral fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	*Dr. Pradhan performed experiments involving statin and JAK2 inhibitor treatment of cells.*
Funding Support:	*NCI*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

-Nothing to Report

What other organizations were involved as partners?

- **Organization Name:** Memorial Sloan Kettering Cancer Center
- **Location of Organization:** New York, NY
- **Partner's contribution to the projec**t *(identify one or more)*
    - **Financial support;**
    - **In-kind support**
    - **Facilities**
    - **Collaboration** – assisted with *in vivo* experiments.
    - **Personnel exchanges**
    - **Other.**

8. SPECIAL REPORTING REQUIREMENTS:

- None

9. APPENDICES:

- None